Chapter

CIRCULATING VASCULAR ENDOTHELIAL GROWTH FACTOR DURING THE NORMAL MENSTRUAL CYCLE

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ABSTRACT

Background: To investigate whether cycle-related variations in circulating VEGF levels would increase the metastatic potential at specific times during the menstrual cycle.
Methods: VEGF levels in serum and whole blood was evaluated during the normal menstrual cycle in premenopausal women. Determination of the menstrual phase was based on hormonal measurements.
Results: A total of 44 samples was taken of six menstrual cycles. Serum VEGF was inversely related with progesterone levels ($r = -0.6$, $P=0.012$). Throughout the menstrual cycle the serum VEGF decreased indicating that the lowest VEGF level occurred during the luteal phase which is compatible with the inverse relation between serum progesterone and VEGF.
Conclusion: These findings, however, do not suggest that individual VEGF levels can direct optimal timing of surgical intervention in breast cancer.

INTRODUCTION

NUMEROUS studies have indicated that survival in breast cancer patients may be affected by the phase of the menstrual cycle at surgery. Patients who were operated on during the proliferative phase tend to have a worse survival than patients operated on during the luteal phase. This was especially observed in premenopausal women with axillary node involvement without distant metastasis.[1-3] This observation led to the hypothesis that the hormonal milieu at the time of surgery may affect the risk of distant metastasis. Various factors could theoretically be involved in this increased risk, among these are proliferation enhancing factors, but also metastasis facilitating circumstances. Angiogenesis is an absolute requirement of tumor growth and metastasis. Folkman postulated that presentation of metastasis is dictated by a strictly regulated balance between negative and positive angiogenic factors. Once pro-angiogenic factors predominate, micro-metastasis switch to the angiogenic phenotype and grow. Vascular Endothelial Growth Factor (VEGF) is a key angiogenic factor for tumor growth and progression.[4-6] Circulating VEGF is increasingly recognized as a prognostic factor in breast cancer. It has been suggested that the systemic effects of circulating VEGF are compatible with tumor growth enhancement, i.e. metastasis progression, at a distant site of the primary tumour.[7]

The existence of a relation between the menstrual cycle and VEGF levels has been a focus of several studies. In view of the obvious relation between angiogenesis and the menstrual cycle, however, conflicting results have been published.[8,9-12] We therefore evaluated circulating VEGF within the normal menstrual cycle in order to detect cycle-related variations that could explain the metastatic propensity at the time of surgery in relation to the menstrual cycle.

MATERIALS AND METHODS

Venous blood sampling

Six healthy premenopausal women were recruited in the study. Blood samples were taken at 3 – 4 day intervals during the menstrual cycle from day 1 through to day 1 of the following cycle. Each menstrual cycle was divided into a follicular and luteal phase according to the LH peak. Informed consent was obtained, and all sampling had been approved by the local medical ethical committee.

Peripheral venous blood samples were collected in sterile CTAD (sodium Citrate Theophilline Adenosine Dypiridamole) tubes (Becton Dickinson Vacutainer Systems Europe, France).

Whole blood and serum

After blood sampling, whole blood samples were diluted with 2 volumes of PBS. Serum was separated by centrifugation at 3000 x g for 10-15 minutes. Whole blood and serum aliquots were kept frozen at –80˚C until the assays were performed. Before determination of VEGF levels all samples were lysed by freezing and thawing twice.

VEGF and hormone assays

VEGF concentrations were determined using the Quantikine Human VEGF enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc. Minneapolis, MN). This is a solid phase ELISA designed to measure $VEGF_{165}$ levels in cell lysates, serum, whole blood, and plasma. All samples were assayed in duplicate. The minimum detectable dose was 9.0 pg/ml as quoted by the manufacturer.

Follicle Stimulating Hormone (FSH) and Luteinising Hormone were determined by ELISA assay (auto Delphia, Pelkin Elmer). Estradiol and progesterone were measured routinely by an in house radio immunoassay.

Statistical analysis

To evaluate the relation between the day of the menstrual cycle, VEGF, estradiol and progesterone Spearman's rank correlation coefficients were calculated in each subject. Using z-transformation, the mean of the correlation coefficients and their 95% CI were calculated. Comparison of the VEGF levels between the two phases of the menstrual cycle was calculated with the Mann-Withney U-test for non-parametric data.

RESULTS

Six premenopausal women with regular menses, no history of endometriosis or infertility, participated in the study. There was no prior history of any breast disorders, none were using oral contraceptives. Blood samples were taken at 3 to 4 day intervals during the menstrual cycle from day 1 through to day 1 of the following cycle. A total of 44 samples were taken of six menstrual cycles: 26 in the proliferative phase and 28 in the luteal phase.

The median length of the menstrual cycles was 29.5 days and ranged from 22 to 30 days. Progesterone levels increased throughout the menstrual cycle and were significantly higher in the luteal phase as compared to the proliferative phase ($r = 0.71$, $P=0.0003$, Table 1).

Table 1. Relation between the day of the menstrual cycle, VEGF, estradiol and progesterone (Spearman's rank correlation coefficients)

	DAY OF MENSTRUATION	B-VEGF	S-VEGF
Estradiol	r = 0.23 (n.s.)	r = 0.19 (n.s.)	r = –0.41 (*P*=0.12)
Progesteron	r = 0.71 (*P*=0.0003)	r = –0.11 (n.s.)	r = –0.6 (*P*=0.012)
Day of menstruation		r = 0.06 (n.s.)	r = –0.54 (*P*=0.005)

B-VEGF = VEGF in whole blood, S-VEGF = VEGF in serum, n.s. = not significant, r = rho

The median progesterone level in the proliferative phase was 0.8 nmol/L (range: 0.4 – 2.5) and 26 nmol/L (range: 2.9 – 78) in the luteal phase ($P<0.005$, see Table 2). Estradiol levels were not significantly different throughout the menstrual cycle (r = 0.23, not significant (n.s.), see Table 1). The median estradiol level in the follicular phase was 490 pmol/L (range: 100 – 1340) and 250 pmol/L (range: 120 – 790) in the secretory phase (n.s., see Table 2). These findings are consistent with normal ovulatory luteal function.

Table 2. VEGF whole blood and serum levels. Comparison of VEGF levels between the two phases of the menstrual cycle

Menstrual phase	NO. OF SAMPLES	ESTRADIOL PMOL/L	PROGESTERON NMOL/L	B-VEGF PG/ML	S-VEGF PG/ML
Proliferative	26	490 (100 – 1340)	0.8 (0.4 – 2.5)	794.5 (479.0 – 1052.0)	400.0 (275.0 – 582.0)
Luteal	28	250 (120 – 790)	26 (2.9 – 78)	678.0 (481.0 – 1128.0)	359.0 (232.0 – 630.0)
P-value		0.1	< 0.005	0.48	0.09

Data are presented as median (range). P-value by Mann-Whitney U-test.

Whole blood VEGF levels were not significantly different throughout the menstrual cycle and were independent of the levels of estradiol and progesterone (r = 0.19 and r = -0.11 respectively, see Table 1). The median whole blood VEGF level in the proliferative phase was 794.5 pg/ml (range: 479.0 – 1052) and 678.0 pg/ml (range: 481.0 – 1128) in the luteal phase (n.s., see table 2). Serum VEGF levels decreased throughout the menstrual cycle (r = –0.54, P=0.005, see table 1) and showed a significant inverse correlation with progesterone (r = –0.6, P=0.012, see Table 1). In the proliferative phase the median serum VEGF level was 400.0 pg/ml (range: 275.0 – 582.0) and 359.0 pg/ml (range: 232.0 – 630.0) in the luteal phase (n.s., see Table 2).

DISCUSSION

In the present study serum VEGF decreased throughout the menstrual cycle indicating that the lowest VEGF levels occur during the luteal phase, compatible with an inversely relation between serum progesterone and VEGF. The present study therefore provides evidence for cycle-related variations of circulating VEGF levels according to the menstrual phase.

From the early studies of Hrushesky et al. till recent reviews, the influence of timing of surgery for breast cancer in relation to the menstrual cycle has remained highly controversial.[13,14] Meta analysis show survival advantages of 5% to 10% or more for women operated on during the early luteal phase,[15] or provide inconclusive evidence.[16-18] The elucidation of putative biological mechanisms accounting for this relation may add to the relative strength of the hypothesis that menstrual cycle timing impacts breast cancer resection outcome, and whether or not specific recommendations would be indicated for breast surgical oncology. Sex steroids regulate many biological functions potentially relevant to breast cancer resection outcome. These include immune function, aspects of cancer cell division and apoptosis as well as factors relating to blood vessel growth or angiogenesis. Studies into a relation of most known or assumed prognostic factors with menstrual cycle, such as mitotic index, receptor status, nodal status, and tumor size, however, have for the most part been negative.[19] A study of Balsari et al, did suggest a relation between the menstrual cycle and Her2neu status.[20]

Folkman explicated the time scales of metastases presentation after tumor resection on the basis of angiogenesis-based tumor dormancy. He suggested that dormancy of metastases is dictated by a strict balance between negative and positive angiogenic factors. After the removal of the primary tumor this balance may alter. Once proangiogenic factors predominate, micrometastases switch to the angiogenic type and grow. VEGF is potentially important because of its central role as a regulatory molecule in tumor angiogenesis, metastatic propensity and cell adhesion.[21] Elevated levels of circulating VEGF have been described in various types of cancer, with higher levels often found in metastatic disease than in localized disease or progressive disease during treatment.[22,23-25] It has been experimentally demonstrated that the administration of anti-VEGF antibodies inhibit the development of metastasis in experimental animal, suggesting that VEGF might affect metastatic propensity via a systemic or endocrine fashion.[26]

Studies on circulating levels of VEGF from premenopausal women during all menstrual phases have found a lack of cyclicity,[11,12,27] or either an increase[28] or decrease[10] of VEGF in the luteal phase.[12,27] In these studies, simultaneous hormonal measurements were performed to identify the physiological phase of menstruation. In two of these studies serum VEGF was significantly lower in the luteal phase.[8,10] It was therefore suggested that VEGF up-regulation by sex-steroids in the follicular phase of the menstrual cycle could predispose to angiogenic driven progression of tumor metastasis.[8,10] This may explain the worse outcome for patients operated on during the follicular phase. To date circulating VEGF is known to reflect mainly VEGF in peripheral blood cells.[29-31] Plasma does not contain significant quantities of VEGF.[31] VEGF measured in serum is platelet-derived. Due to variations in sample handling, however,

variations in serum VEGF occur and have been reported.[32,33] Consequently differences in published concentrations of circulating VEGF as measured in serum have been reported.[34] This may in part explain the inconsistency of data on VEGF during the menstrual cycle. Salven et al suggested measurements in whole blood, which reflects all cell fractions and the negligible amount of VEGF in the plasma, to be a more reliable indicator for circulating VEGF than serum VEGF.[31] We measured VEGF in serum and whole blood. Although we confirmed the relation between cycle stage and VEGF levels, and especially between progesterone and VEGF levels,[10] we found no differences in the median serum and whole blood VEGF levels, between the two phases. It is therefore unlikely that selection of timing of surgery in relation to VEGF levels on an individual base would influence the individual prognosis.

A recent study of Graubert et al stressed a new insight into the regulation of VEGF-mediated angiogenesis during the physiologic menstrual cycle. This study demonstrated in human endometrium that functional VEGF signaling, as assessed by KDR receptor phosphorylation studies, was active in the late menstrual, and early proliferative phases. KDR phosphorylation inversely correlated to the presence of soluble Flt-1 (sFlt-1). In this study determination of the phase of menstruation was made by endometrial sampling which is a very reliable method to determine the cycle stage.[35] sFlt-1 acts as sink molecule rendering less VEGF molecules available for binding with the KDR receptor. In addition it also acts as a dominant negative regulator by forming inactive heterodimers with transmembrane receptors. According to the data of Graubert, sFlt-1 decreases during the follicular phase, this would indicate that VEGF is unopposed to induce neovascularization during the follicular phase. The findings of Graubert et al therefore indicate that, although we found no significant differences in circulating VEGF between the two phases of the menstruation cycle, VEGF-mediated angiogenesis may still be responsible for a metastases facilitating milieu during the follicular phase via a decreased regulation by sFlt-1of VEGF's action on KDR activation.

Despite the controversial data, reported survival advantages of optimally timed resection approach the benefit achieved by adjuvant chemotherapy.[36] So far however, together with the present findings, studies on the relation between circulating VEGF and the normal menstrual cycle do not suggest that VEGF levels can direct optimal timing of surgical intervention in breast cancer.

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